# Acute hypoxia modifies regulation of neuroglobin in the neonatal mouse brain

Hümmler, Nicolas ; Schneider, Christina ; Giessl, Andreas ; Bauer, Reinhard ; Walkinshaw, Gail ; Gassmann, Max ; Rascher, Wolfgang ; Trollmann, Regina

Abstract: Among endogenous adaptive systems to hypoxia, neuroglobin, a recently discovered heme protein, was suggested as a novel oxygen-dependent neuroprotectant. We aimed to characterize i) maturational age-related regulation of neuroglobin in the developing mouse brain under normoxic and hypoxic conditions, and ii) the role of hypoxia-inducible transcription factors (HIFs) as possible mediators of O(2)-dependent regulation of neuroglobin in vitro and in vivo. During early stages of postnatal brain maturation (P0-P14) neuroglobin mRNA levels significantly increased in developing mouse forebrains. By immunohistochemical analysis we confirmed expression of neuroglobin protein in the cytoplasm of developing neurons but not glial cells under normoxic conditions. Exposure of the immature brains (P0, P7) to acute (8% O(2), 6h) and chronic systemic hypoxia (10% O(2), 7days) led to differential activation of neuroglobin varying with maturational stage (P0, P7) and severity of hypoxia. This observation may indicate that neuroglobin is involved in adaptive responses of immature neurons to acute hypoxia during an early stage of mouse brain maturation (P0). In response to activation of the HIF system by prolyl-4-hydroxylase inhibitor (FG-4497), neuroglobin mRNA expression was significantly up-regulated in primary mouse cortical neurons (DIV6) exposed to normoxia and hypoxia (1% O(2)) compared to non-treated controls. In conclusion, present results strongly indicate that cerebral regulation of neuroglobin is related to maturational stage and that hypoxia-induced neuroglobin up-regulation is modified by the HIF system.

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# Acute hypoxia mediates expression of neuroglobin in the neonatal mouse brain

Abbreviated title: Neuroglobin expression in neonatal mouse brain

**Nicolas Hümmler[1], Christina Schneider[1], Andreas Giessl[2], Reinhard Bauer[3], Gail Walkinshaw[4], Max Gassmann[5], Wolfgang Rascher[1], Regina Trollmann[1,5]**

[1]Department of Pediatrics and [2]Institute of Animal Physiology, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany

[3]Institute of Physiology, University of Jena, Germany

[4]FibroGen Inc., San Francisco, California

[5]Institute of Veterinary Physiology, Vetsuisse Faculty, and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Switzerland

**Corresponding author:**
Regina Trollmann, MD
Department of Pediatrics, Friedrich-Alexander-University of Erlangen-Nuremberg
Loschgestrasse 15, 91054 Erlangen, Germany
*Tel: ++49/9131/853-3118; Fax: ++49/9131/853-3113*
E-mail: regina.trollmann@uk-erlangen.de

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**Abstract**

Among endogenous adaptive systems to hypoxia, neuroglobin has been suggested as a novel oxygen-dependent neuroprotectant. However, neuroglobin´s protective role in postnatal susceptibility to hypoxic brain injury, as well as the mechanism underlying hypoxic regulation of neuroglobin, remains to be elucidated.

We aimed to characterize i) maturational age-related regulation of neuroglobin in developing mouse brain in normoxic and hypoxic conditions, and ii) the role of hypoxia-inducible transcription factors (HIFs) as possible mediators of $O_2$-dependent regulation of neuroglobin.

In normoxia we showed that neuroglobin expression is regulated postnatally with increasing neuroglobin mRNA levels observed during brain maturation (P0-P14). By immunohistochemical analysis, we observed that neuroglobin is localized mainly in the cytoplasm of neurons. Exposure to acute (8% $O_2$, 6h) and chronic systemic hypoxia (10% $O_2$, 7d) revealed that the neuroglobin response to hypoxia varies with maturational stage (P0 vs. P7) as well as with the severity of hypoxia, indicating a role for neuroglobin in neuronal acclimatisation to acute hypoxia during early stages of mouse brain maturation (P0). We also investigated neuroglobin regulation in primary mouse cortical neurons (DIV6) exposed to hypoxia (1% $O_2$) in the presence or absence of the novel HIF prolyl hydroxylase inhibitor, FG-4497. The data generated strongly supports the notion that hypoxia-induced neuroglobin up-regulation is HIF-dependent.

**Key words**

Hypoxia-inducible transcription factor, Bnip3, immediate early genes, hypoxia-induced apoptosis

## Introduction

Hypoxic and ischemic complications during the pre- and perinatal period are common causes of acquired neonatal brain damage (Rutherford et al., 2006). Clinical situations representing risk factors for perinatal brain injury include maternal hypotension or anemia, placental or umbilical cord complications (Low, 2004), as well as chronically compromised prenatal fetal oxygen supply due to placental abnormalities or maternal diseases (Terzidou and Bennett, 2001). The complex neurotoxic cascade following cerebral hypoxic injury develops over a specific period of time when immediate early genes, inflammatory and excitotoxic pathways and, finally, apoptotic neurodegeneration are activated in vulnerable CNS regions such as cerebral cortex, hippocampus (CA1, CA3) and striatum (Northington et al., 2001).

Among endogenous cerebral adaptive systems to hypoxia and ischemia, neuroglobin (Ngb), a recently discovered heme protein expressed in neurons of the central and peripheral nervous system and retina was suggested as a new neuroprotectant which is regulated by oxygen levels. *In vitro*, hypoxia has been shown to up-regulate Ngb transcript and protein expression in mouse cortical neurons (Sun et al., 2001), human glioblastoma (Emara et al., 2009) and neuronal cell lines (Schmidt-Kastner et al., 2006, Shao et al., 2009)). Moreover, increased Ngb expression in hypoxic adult rat brains (Li et al., 2006, Shao et al., 2009) as well as in focal cerebral ischemia (Sun et al., 2001) has been demonstrated. However, the *in vivo* observations are contradictory (Hundahl et al., 2005, Li et al., 2006, Schmidt-Kastner et al., 2006) with findings varying depending on the experimental model used. Notably, studies on Ngb expression and regulation in developing brain, as well as its role in early response to cerebral hypoxia, are not available.

Mechanisms underlying hypoxic induction of Ngb are still unclear. From *in vitro* experiments, involvement of hypoxia-inducible transcription factors (HIFs) (Sun et al., 2001) and hemin via the soluble guanylate cyclase/protein kinase G pathway (Zhu et al., 2002) have been proposed. Expression of Ngb was shown to be significantly induced in mouse cortical

neurons by $CoCl_2$ and deferoxamine (DFO) (Sun et al., 2001). This compounds can stabilize the $O_2$-regulated α-subunits of HIF which then heterodimerize with the constitutively expressed β-subunit to form an active HIF complex (Fandrey et al., 2006). Under normoxic conditions, HIF-α subunits are degraded rapidly by the ubiquitin-proteasome pathway, a process that is mediated via hydroxylation of specific prolyl residues by an enzyme family of HIF prolyl hydroxylases (prolyl hydroxylation domain protein [PHD]) that utilize di-oxygen and 2-oxoglutarate as co-substrates. Reduced activity of the PHDs, e.g. at low oxygen levels or by iron chelation, results in stabilisation of the HIF-α protein, heterodimerization (α/β), and nuclear translocation followed by binding to hypoxia response elements in promoters of specific responsive genes. HIFs up-regulate transcription of a cascade of hypoxia-inducible genes including erythropoietin, pro-apoptotic mediators (e.g. BNIP3), and vasoactive factors such as vascular endothelial growth factor (VEGF) and adrenomedullin (ADM), all of which can modify immediate cerebral response to hypoxia and ischemia in the adult (Stroka et al., 2001) as well as developing brain (Bernaudin et al., 2002, Trollmann et al., 2008b). Based on these observations, involvement of HIF-1 in the hypoxic induction of cerebral Ngb expression has been hypothesized (Sun et al., 2001).

To analyze HIF stabilization using a pharmacological agent that is more specific than $CoCl_2$ and DFO, we have used a novel prolyl-4-hydroxylase inhibitor, FG-4497 (Schneider et al., 2009). FG-4497 inhibits the HIF-PHDs, stabilizes HIF-α subunits, and thus enhances formation of a functional HIF complex.

Functionally, Ngb has been shown to protect neurons from hypoxic injury *in vitro* (Liu et al., 2009) and *in vivo* in adult wild-type mice (Shao et al., 2009) and in Ngb-overexpressing transgenic mice (Sun et al., 2003). The protective properties of Ngb may be due to its ability to act as scavenger of reactive oxygen and nitrogen species (Brunori et al., 2005, Fordel et al., 2007) and to maintain mitochondrial function (Liu et al., 2009, Yu et al., 2009). Furthermore,

anti-apoptotic effects of Ngb via reduction of cytochrom c have been reported (Fago et al., 2006, Raychaudhuri et al., 2009). The role of Ngb in age-related susceptibility to hypoxic/ischemic brain injury remains to be established.

The aim of the present study was to characterize maturational postnatal expression of Ngb in developing mouse brain during normoxia and systemic hypoxia using a mouse model of acute and chronic perinatal hypoxia (Trollmann et al., 2008a). In addition, *in vitro* and *in vivo* experiments were performed to understand the role of HIF in hypoxia-induced induction of Ngb.

**Methods**

**Animal experiments.** Neonatal C57BL/6 wild-type mice (Charles River Laboratories, Germany) were exposed to acute and chronic systemic hypoxia using a closed hypoxia chamber (Hypoxic Workstation INVIVO$_2$ 1000, Biotrace International, UK). For this purpose, neonatal mice were kept at continuous systemic hypoxia with $FiO_2$ of 8% for 6 h (*acute hypoxia group*) at postnatal day 0 (P0, n=30) and P7 (n=30). To analyze effects of *chronic systemic hypoxia,* 7-day-old mice (P7) were kept under continuous systemic hypoxia with $FiO_2$ of 10% for 7 d (n=30). To enable adjustment to the hypoxic environment, $O_2$ deprivation was done gradually by decreasing the $FiO_2$ in 2 % $O_2$ steps every 10 min. Controls (neonatal mice, n=30 per group) were kept in the INVIVO$_2$ chamber under room air. For reoxygenation experiments, neonatal mice were kept in the chamber at room air (21% $O_2$) for 24 h, 72 h or 7 d together with their dams (to allow for normal temperature and nutrition). Animal experiments were performed according to protocols approved by local government (Regierung Mittelfranken, Germany) according to national and European law.

After the incubation period, neonatal brains were immediately dissected (without reoxygenation of the hypoxic groups), frozen in liquid nitrogen and stored at -80 °C until protein (n=3 per group) and mRNA extraction (n=5 per group) was performed. For

immunohistochemical studies, brains (n=3 per group) were embedded in 4% paraformaldehyde. Coronal sections at the level of the dorsal hippocampus were examined to analyze parietal cortex and hippocampus known to show selective vulnerability to hypoxia at early development. Mouse brain maturation at P0 and P7 approximately corresponds to that of the human brain at mid-gestation and near-term, respectively (Dobbing and Sands, 1979).

**Pharmacological HIF stabilization.** Neonatal C57BL/6 wild-type mice were treated with the HIF prolyl hydroxylase inhibitor (PHI) FG-4497 (FibroGen; San Francisco; USA) under normoxic conditions at P0 (n=15) or P7 (n=15). FG-4497 (patent-registered US20040254215A1) was dissolved according to the instructions of the manufacturer (100 mg FG-4497 to 9.675 ml of 5% Dextrose to 325 µl of 1 N NaOH), and was injected i.p. (injection volume 0.1 ml) in a single dose of 30 (n=5), 60 (n=5) and 100 mg/kg (n=5). Age-matched controls were treated with NaCl 0.9% i.p. (n=10) or remained non-treated (n=10). After the treatment period of 6 h, PHI-treated brains and controls were dissected, frozen in liquid nitrogen and stored at -80°C until mRNA extraction. A 6-hour treatment interval was decided according to observations on protective effects of other PHI than FG-4497 *in vivo* in adult rats (Siddiq et al., 2005, Bernhardt et al., 2006). Specificity of FG-4497 has been shown in adult and neonatal mouse brain (Schneider et al., 2009).

**Primary cortical neuronal cultures**. The dorsal telencephalon was dissected from E14 C57BL/6 wild-type mice (Charles River Laboratories, Germany) following a published protocol (Sestan et al., 1999). Briefly, cortices of E14 mouse pups were dissected aseptically in cold 1x HBSS (+) solution with $MgCl_2$ and $CaCl_2$ following decapitation. The meninges were removed, cortices were collected in 1x HBSS (+) and after washing in 1x HBSS (-) without $MgCl_2$ and $CaCl_2$ but with Pyruvate were treated with trypsin DNAse. After washing in DMEM solution with Pyruvate and fetal calf serum, tissues were dissociated by triturating in serum free media to make a single cell suspension. The serum free medium (DMEM+Glutamax) was supplemented with B-27 supplement, Albumax, Sodium Pyruvate

and penicillin–streptomycin. Arabinoside ($2\times10^{-5}$ M) was used for the inhibition of astrocyte multiplication. Cells were plated at a density of $5\times10^3$ cells/cm$^2$ onto plastic Petri dishes coated with poly-L-lysine in borate buffer, and were incubated with the serum free media for 6 d (DIV6) in normal atmosphere with 5% $CO_2$. At DIV6, cultures were incubated with FG-4497 (5, 30, 60, 200 μM) for 6 h in normoxic (21% $O_2$) or hypoxic (1% $O_2$, 5% $CO_2$, 94% $N_2$) conditions. Cell viability was assessed by lactate dehydrogenase measurement. Experiments were performed at least three times. Cells were lysed and probed for HIF-1α by western blot analysis as previously described (Trollmann et al., 2008b). Blots were quantified desitometrically using the MCID imaging system (Imaging Research). β-actin was used as a loading control.

**RNA isolation and RT-PCR.** Total RNA was extracted from whole mouse brains and primary cortical neurons using Trizol isolation method (Invitrogen, Germany). RT-PCR was performed as described previously (Trollmann et al. 2008a, b). One μg of total RNA was used for each reverse transcription (MMLV reverse transcriptase, Invitrogen, Germany). RT cDNA products were measured by quantitative TaqMan RT-PCR. Commercial reagents (TaqMan PCR Reagent Kit, Perkin-Elmer Corp.) and conditions were applied according to the manufacturer´s protocol. The PCR reaction was performed in an ABI 7500 real-time PCR thermocycler (Applied Biosystems). All reactions were performed in duplicate using ß-actin and porphobilinogen deaminase (PBGD) as endogenous controls.

The following primers and TaqMan probes based on published reports were used:

*ß-actin*, forward: 5´- ATGCTCCCCGGGCTGTAT - 3´;
reverse: 5´- TCACCCACATAGGAGTCCTTCTG - 3´;
TaqMan probe: 5´(FAM) - ATCACACCCTGGTGCCTAGGGCG - (TAMRA) - 3´;
*ADM*, forward: 5´- TGGACGAGCAGAACACAACTG - 3´;
reverse: 5´ - CTGGCGGTAGCGTTTGACA - 3´;
TaqMan probe: 5´(FAM) – CCCTACAAGCCAGCAATCAGAGCGAA - (TAMRA) - 3´;
*BNIP3*, forward: 5´- ATTCCCCCCAAGGAGTTCCT – 3´;
reverse: 5´- TTCATAACGCTTGTGTTTCTCATG – 3´;
TaqMan probe: 5´(FAM) – ACACCCGAAGCGCACAGCTACTCTCA – (TAMRA) – 3´;

*DUSP1*, forward: 5′- TCCTAACCACTTTGAGGGTCACTAC - 3′;
reverse: 5′ - CAGCATCCTTGATGGAGTCTATGA - 3′;
TaqMan probe: 5′(FAM) – ACAAGAGCATCCCTGTGGAGGACAACC - (TAMRA) - 3′;
*EPO*, forward: 5′- AAGGTCCCAGACTGAGTGAAAATATTAC - 3′;
reverse: 5′ - GGACAGGCCTTGCCAAACT - 3′;
TaqMan probe: 5′(FAM) – TCTATGGCCTGTTCTTCCACCTCCATTCT - (TAMRA) - 3′;
*IER3*, Gene expression assay (Applied Biosystems; Germany; Mm00519290_g1)
*NGB*, forward: 5′ - ATCGGGCAGTGGGAGTGA - 3′;
reverse: 5′ - CAGGCACTTCTCCAGCATGTAG – 3′;
TaqMan probe: 5′(FAM) – TCCTTCTCGACAGTAGGCGAGTCCCTG – (TAMRA) – 3′;
*PBGD,* forward: 5′- ACAAGATTCTTGATACTGCACTCTCTAAG - 3′;
reverse: 5′- CCTTCAGGGAGTGAACAACCA - 3′;
TaqMan probe: 5′(FAM) - TCTAGCTCCTTGGTAAACAGGCTCTTCTCTCCA - (TAMRA)-3′;
*PHD2*, forward: 5′- AAGCTGGGCAACTACAGGATAAA - 3′;
reverse: 5′ - CGTGACGGACATAGCCTGTTC - 3′;
TaqMan probe: 5′(FAM) – ACGAAAGCCATGGTTGCTTGTTACCCA - (TAMRA) - 3′;
*PHD3*, forward: 5′- AGCCCATTTTTGACAGACTTCTG - 3′;
reverse: 5′ - AGCGTACCTGGTGGCATAGG - 3′;
TaqMan probe: 5′(FAM) – TCTGGTCAGACCGCAGGAATCCACAT - (TAMRA) - 3′;

**Fluorescence immunhistochemistry.** For immunohistochemical analysis sections (3 μm) of paraformaldehyde-embedded brain tissues were used. After heat-induced epitope retrieval (Citrate buffer, pH 6, 10mM, S2369, DAKO, Germany), washing (PBS/Triton-X 0.1%) and blocking with Protein Block (X0909, Serum Free, DAKO, Germany) sections were incubated with the polyclonal rabbit anti-Ngb antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:100) over night at 4 °C. Negative controls were performed by omitting the primary antibody. After washing sections were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Eugene, USA) secondary antibody for 60 min. After washing sections were co-stained with monoclonal mouse anti-GFAP (glial fibrillary acidic protein) antibody (1:50; Santa Cruz Biotechnology) to identify glial cells, or monoclonal mouse anti-NeuN (neuronal nuclei) antibody (Chemicon Int. Hampshire, UK, 1:200), a routinely used neuronal marker, at room temperature for 2 h, followed by Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (Invitrogen, Eugene, USA). Cell nuclei were stained with DAPI (4′,6-diamidino-2-phenylindol). During all incubation periods sections were stored in a

humidified chamber. Coverslips were mounted with Gel Mount Aqueous Mounting Medium (Sigma, St. Luis, USA) and air-dried.

**TUNEL staining.** To determine degree of apoptosis-like cell death, terminal deoxynucleotidyl transferase-mediated dUTP end-labeling (TUNEL) staining (In Situ Cell Death Detection Kit, POD; Roche, Germany) of coronal sections at the level of dorsal hippocampus was performed 72 h and 7 d after systemic hypoxia (n=3 per group). The number of TUNEL-positive cells in parietal cortex and hippocampus (CA1) was then counted and compared to normoxic controls [3-6 fields/region per section were examined using the Olympus microscope BX51 (Tokyo, Japan)].

*Statistical analysis*. Data are expressed as mean±SEM. Statistical significance was determined by One-way ANOVA and Two-way ANOVA for repeated measurements ($p$-value<0.05).

## Results

**Cerebral Ngb expression is related to maturational stage.** To evaluate maturational age-related expression levels of Ngb during postnatal mouse brain development, we measured cerebral Ngb mRNA levels in normoxic brains of mice from 0 to 21 days of postnatal age and at P60. As demonstrated in Fig. 1, Ngb mRNA levels increased during the first postnatal weeks from P0 to P14 reaching peak levels at P14, and declined thereafter reaching levels comparable to those of adult brains (P60) at the end of third postnatal week. Immunohistochemical analysis revealed neuronal but not glial expression of Ngb throughout the developing cerebral cortex at P7 (Fig. 2). Of note, Ngb-positive neurons were found most prominently in cortical layers II/III.

**Cerebral Ngb expression is related to the degree of hypoxia.** To investigate effects of hypoxia on cerebral Ngb expression during early postnatal brain development, we analyzed Ngb mRNA and protein expression in brains of neonatal mice at the age of P0 and P7 exposed to acute or chronic hypoxia. Whereas acute systemic hypoxia (8% $O_2$) for 6 hours did not markedly alter cerebral Ngb mRNA levels at P7, there was a significant up-regulation of Ngb mRNA concentrations at P0 compared to controls (Fig. 3A). However, hypoxia-induced Ngb up-regulation did not persist during reoxygenation (21% $O_2$ for 24 h, 72 h, and 7 d) following exposure to acute hypoxia (Fig. 3B). The increase of cerebral Ngb mRNA concentrations with postnatal age in normoxic brains (Fig. 3B) reflects an age-related phenomenon as shown in Fig. 1. Interestingly, this age-specific up-regulation of Ngb appeared to be transiently suppressed upon acute hypoxia (Fig. 3B) as suggested by unchanged mean Ngb mRNA levels during the first 72 h of reoxygenation in contrast to normoxic controls.

To confirm the severity of acute hypoxia used in our experimental setting, several hypoxia-inducible genes as well as markers of apoptosis were analyzed (Fig. 4 A-G). At both maturational stages P0 and P7, mRNA expression of HIF-regulated vasoactive and neurotrophic factors (ADM, EPO) and pro-apoptotic genes (BNIP3, IER3) was significantly

increased compared to normoxic controls with no apparent age-related differences. Similarly, cerebral mRNA concentrations of the HIF-dependent PHD2 and PHD3 isoforms (Fig 4E, F) were up-regulated during acute hypoxia compared to normoxic controls in both P0 and P7 brains. In contrast, mRNA levels of the proapoptotic HIF target gene DUSP1 were significantly elevated in hypoxic developing brain at P0 compared to controls but not at P7 (Fig. 4G) indicating age-related differential regulation of DUSP1 by hypoxia. Functional activation of cerebral apoptosis following acute systemic hypoxia (8% $O_2$, 6h) was confirmed by TUNEL staining of forebrain sections upon reoxygenation periods of 24 h, 72 h and 7 d (Fig. 5) which demonstrated increased numbers of TUNEL+ cells in parietal cortex (Fig. 5 B, H) and hippocampus (Fig. 5 D).

Since acute hypoxia was found to be a stimulus for Ngb induction during early brain development, cellular and spatial distribution of Ngb in hypoxic developing brains was determined by immunohistochemistry (Fig. 6). A hypoxia-induced increase in Ngb at P0 was confirmed at the protein level by demonstrating a markedly higher number of Ngb-positive cells in the parietal cortex of hypoxic compared to normoxic mouse brains (Fig. 6 A vs B). This was in contrast to hypoxic mouse brains at P7, in which there was an unchanged number and distribution pattern of Ngb-positive cells compared to controls (Fig. 6 C vs D). Notably, co-staining with NeuN and GFAP demonstrated cytoplasmic accumulation of Ngb in hypoxic neurons (Fig. 2C, D) but not glia cells (Fig. 2B) throughout the developing cerebral cortex.

To determine whether Ngb expression is also modulated by chronic hypoxia during early stages of brain maturation, analyses were extended to brains exposed to systemic chronic hypoxia at P7 (10% $O_2$ for 7 d). As demonstrated in Table 1, cerebral Ngb mRNA levels did not significantly differ between chronic hypoxic and normoxic tissues either at the end of hypoxic exposure or during reoxygenation periods. To compare Ngb mRNA levels to other HIF-regulated genes, we determined mRNA concentrations of BNIP3, ADM, EPO, PHD2 and PHD3, DUSP1 and IER3. In contrast to our observations during acute hypoxia, there

were no significant differences in selected HIF target gene expression between normoxic and hypoxic brain except for a significant EPO up-regulation, and, of note, a more than 2-fold increase of BNIP3 mRNA levels following a 24 hr reoxygenation period in comparison to normoxic controls (Table 1).

**Hypoxic induction of cerebral Ngb expression is mediated by HIF.** To examine whether hypoxia-induced up-regulation of Ngb expression is mediated by HIF stabilization and activation during acute hypoxia, two approaches were taken. First, by *in vitro* experiments with mouse embryonic neuronal cultures (E14, DIV6) we compared induction of Ngb mRNA levels between neurons exposed to hypoxia (1% $O_2$, 6h) and in neurons pre-treated with FG-4497 (0.1-1 μM) before incubation under either normoxic or hypoxic conditions. Hypoxia induced a more than three-fold increase in Ngb mRNA levels compared to controls (Fig. 7A). Similar transcriptional activation of Ngb was found in response to FG-4497 under normoxia (Fig. 7A) indicating HIF-dependent regulation of Ngb at the mRNA level. Notably, Ngb mRNA levels did not significantly vary between cells grown in hypoxic and hypoxic+FG-4497 conditions, suggesting no additive effects of hypoxia and PHI treatment. In addition, FG-4497-induced HIF-1α stabilization in these neuronal cultures was verified by western blot analysis (Fig. 7B), revealing prominent HIF-1α protein accumulation under normoxia at concentrations of 30-60 μM. Once again, there were no additive effects of hypoxic+FG-4497 conditions compared with hypoxic controls (Fig. 7B). Conversely, additive effects of hypoxia and FG-4497 are observed for the HIF-dependent gene, ADM (Fig. 7D). BNIP3 mRNA levels on the other hand, increased in response to hypoxia and FG-4497 without comparable additive effects (Fig. 7C).

The second approach taken was to analyze cerebral Ngb expression *in vivo* in response to PHI treatment (30, 60 and 100 mg/kg, i.p.) under normoxic conditions (Table 2). No significant difference in mean Ngb mRNA expression level was detected between vehicle-treated and FG-4497-treated neonatal mouse brains at both maturational stages P0 and P7. In addition,

FG-4497 treatment did not significantly alter mRNA levels of the HIF-regulated BNIP3 in neonatnal mouse brain at either P0 or P7.

**Discussion**

A wide spectrum of endogenous hypoxia-inducible mediators are involved in critical mechanisms of early brain maturation (Fandrey et al., 2006) as well as compensation during hypoxic and ischemic injury of the developing brain (Curristin et al., 2002). Here, we show that Ngb expression develops age-dependently with increasing Ngb levels during early postnatal mouse brain maturation (P0-P14), and that Ngb is localized mainly in the cytoplasm of neurons at this early stage of brain development. Further, using a neonatal mouse model of systemic hypoxia we demonstrate that Ngb response to hypoxia varies with maturational stage and degree of hypoxia. Another major finding of the present study is that hypoxia-induced Ngb up-regulation is HIF-dependent as shown *in vitro* by pharmacological HIF stabilization.

*Cerebral Ngb expression is related to maturational stage.* Focusing on physiological expression of Ngb during mouse brain development, we report for the first time a developmental transient increase of Ngb expression at the end of the 2nd postnatal week. Thus, present data extend previous findings on age-specific regulation of Ngb expression which demonstrated an age-related decline in 3 to 12-month-old rodents (Sun et al., 2005). Previous studies on adult mouse brain have suggested multi-regional expression of Ngb (Wystub et al., 2003), whereas others have not been able to detect either Ngb-positive neurons (Mammen et al., 2002) or glia cells (Hundahl et al., 2005) in the normoxic adult mouse cerebral cortex and hippocampus. Although spatial expression and functional role of Ngb during early development are not yet well defined, increasing levels of Ngb mRNA from P0 to P14 in developing mouse forebrain as shown here may indicate an important role of Ngb in the regulation of crucial processes of early postnatal mouse brain development. Indeed, neurons of the developing mouse cerebral cortex representing a metabolically active region during early brain maturation were found to highly express Ngb under normoxic conditions. The ability of Ngb to modify $O_2$ homeostasis by mitochondrial pathways (Raychaudhuri et al.,

2009) might improve early cell survival, neurogenesis and, finally, brain plasticity. In accordance with others (Sun et al., 2003, Hundahl et al., 2005) we did not observe co-expression of Ngb with GFAP suggesting that Ngb does not play a major role in early postnatal glial development.

*Cerebral Ngb expression follows the degree of systemic hypoxia and stage of maturation.* The severity of acute brain hypoxia (8% $O_2$, 6h) induced in our experimental setting is supported by significant induction of apoptosis in vulnerable brain regions shown by TUNEL staining. In addition, transcriptional up-regulation of pro-apoptotic genes, namely BNIP3 (Zhang et al., 2007), IER3 and DUSP1, indicates activation of cerebral pro-apoptotic mechanisms by early postnatal exposure to acute hypoxia (8% $O_2$, 6h). Additionally, the consistent increase of HIF-regulated vasoactive genes (ADM), neurotrophic genes (EPO) and PHDs in response to acute hypoxia compared to normoxia indicates activation of the HIF-system due to cerebral oxygen deprivation at P0 and P7. Of note, we have previously shown HIF-1$\alpha$ and HIF-2$\alpha$ activation in postnatal mouse brain at P0 and P7 in response to acute systemic hypoxia (8% $O_2$, 6h) (Trollmann et al., 2008a).

At early stage of mouse brain maturation (P0), acute but not chronic hypoxia increased cerebral Ngb expression as demonstrated by increased mRNA levels in forebrain homogenates and higher numbers of Ngb-positive neurons throughout hypoxic developing cerebral cortex compared to normoxic controls. Of note, the rapid decline of Ngb mRNA levels observed during reoxygenation indicates a transient up-regulation of Ngb during the early response to acute hypoxia - this was found exclusively during early development (P0) but not at P7. In contrast, studies on adult mice showed no detectable difference in cerebral Ngb expression between normoxic and hypoxic brains either at the protein or mRNA levels upon short-term hypoxia (7.6% $O_2$, 2h) (Hundahl et al., 2005). In accordance with PCR and ISH studies on adult mouse brain under chronic hypoxic conditions (10% $O_2$; up to two

weeks) (Mammen et al., 2002) we did not detect any differences in Ngb expression between chronically hypoxic brains and normoxic controls either at P0 or at P7, even with reoxygenation periods up to 7d (data not shown). Thus, present results suggest that Ngb transcriptional regulation is affected by *acute* but not by chronic hypoxia, and interestingly, that acute hypoxic induction of cerebral Ngb represents an age-dependent, transient phenomenon during early brain development.

***Hypoxic induction of cerebral Ngb expression is mediated by HIF.*** PHI treatment allows the analysis of HIF stabilization as well as HIF target gene activation under normoxic conditions (Paliege et al., 2010, Robinson et al., 2008, Rosenberger et al., 2008). Recent studies from our group have shown that a novel PHI, FG-4497, can penetrate the blood-brain-barrier and activate vasoactive and neurotrophic HIF target genes in a dose-dependent manner in developing mouse brain at P7 (Schneider et al., 2009). Expanding on these previous observations, in the current study we demonstrate an increase in Ngb transcription in neuronal cells in response to FG-4497 treatment. This *in vitro* data suggests that the hypoxic regulation of Ngb mRNA expression is HIF-dependent. However, FG-4497-induced regulation of Ngb expression could not be confirmed *in vivo* in developing mouse brain at P0 and P7. As developmentally different regulation has to be considered, further *in vivo* investigations at different maturational stages are necessary. Additionally, differential regulation and sensitivity of HIF target genes *in vivo* (Trollmann et al., 2008a) might be another possible explanation of contradictory *in vitro* and *in vivo* observations.

Although there are conflicting hypotheses about the potential effects of PHI treatment in neonatal brain (Chen et al., 2009), pharmacologic inhibition of PHD hydroxylation leading to HIF accumulation has been proposed as a possible target for neuroprotection (Siddiq et al., 2005, Adamcio et al., 2009)). *In vitro*, HIF stabilization by FG-4497 has been shown to enhance proliferation, neurogenesis and differentiation of human neural progenitor cells

(Milosevic et al., 2009). In contrast, depending on the degree of hypoxia and the cell type induction of pro-apoptotic mechanisms has been linked to overexpression of HIF (Helton et al., 2005, Chen et al., 2009). This is of special significance in the developing brain as since disturbance of physiological apoptosis which plays a crucial role in early brain development (Sandau and Handa, 2006), might be detrimental. In accordance to reported evidence that HIF-1 induces BNIP3 expression (Guo et al., 2001), we have shown a significant up-regulation of cerebral BNIP3 mRNA levels in response to FG-4497 *in vitro* under normoxic conditions. Notably, this was not confirmed *in vivo*. BNIP3 is known as a central mediator of hypoxia-induced autophagy that could result in adaptive survival as well as cell death depending on the degree of hypoxia (Tracy et al., 2007, Bellot et al., 2009). In contrast to severe hypoxic conditions or anoxia (<0.1% $O_2$) which trigger HIF-independent autophagic response, sublethal hypoxia (as used in our model) induces mitochondria-associated proteins BNIP3 and BNIP3L (NIX) which have been suggested to mediate a metabolic adaptation for survival by their ability to control reactive oxygen species production and DNA damage (Bellot et al., 2009). Of interest, Aminova et al. (2008) showed that antioxidants and PHD inhibitors prevent HIF-mediated transactivation of BH3-only proteins minimizing pro-death effects of HIF-1α overexpression on neurons exposed to oxidative stress. We intend to address functional significance of early Ngb versus BNIP3 induction by pharmacological HIF overexpression in the hypoxic developing mouse brain in future studies.

In conclusion, our *in vivo* results demonstrate for the first time that Ngb expression increases age-dependently in the developing mouse brain and that Ngb expression in response to hypoxia varies with maturational stage as well as the degree of hypoxia, indicating a role in neuronal adaptation to acute hypoxia during early stage of mouse brain maturation (P0). Regarding hypoxic regulation of Ngb, our *in vitro* results suggest involvement of HIF-dependent mechanisms. Taken together, our data provide basic information for further

investigations on pathophysiology of postnatal cerebral hypoxic response in view of novel and maturational-age specific neuroprotective concepts.

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## Legends to Figures and Tables

**Fig. 1** Ngb mRNA expression normalized to β-actin in mouse brain in relation to maturational age P0-P60 . N=4 per group, **p<0.01

**Fig. 2** Immunohistochemical analysis of Ngb in normoxic mouse cerebral cortex at P7. Merged photomicrographs of Ngb detected with Alexa Fluor 488, *green* and GFAP detected with Alexa Fluor 594, *red* are shown in **A** and **B**, and of Ngb and NeuN detected with Alexa Fluor 594, *red* in **C** and **D**. **A** Overview of parietal cortex. Co-staining of Ngb with GFAP demonstrating Ngb+ cells most prominently in cortical layers II and III. **B** Higher magnification did not reveal Ngb expression in GFAP+ glial cells. **C, D** Co-staining of Ngb with NeuN showing localisation of Ngb in neurons.

**Fig. 3 A** Maturational stage-dependent up-regulation of Ngb mRNA levels normalized to PBGD in developing mouse brain P0, P7 in response to acute hypoxia 8% $O_2$; 6h compared to normoxic controls, N=5 per group. **B** Transient down-regulation of Ngb mRNA levels in hypoxic developing mouse brains at P0 during reoxygenation R compared to normoxic brains. R, reoxygenation intervals of 24h, 72h and 7d. N=3 per group. **p<0.01

**Fig. 4** Up-regulation of cerebral mRNA levels of HIF-dependent target genes in response to acute systemic hypoxia at P0 and P7 compared to normoxia. Independently from maturational stage mRNA concentrations of BNIP3 **A** , ADM **B** , IER3 **F** , EPO **G** and PHD isoforms **C**, **D** significantly increased during acute hypoxia compared to normoxia. **E** Differential regulation of DUSP1 mRNA expression in relation to maturational stage in response to acute hypoxia compared to controls. N=5 per group, *p<0.05; **p<0.01

**Fig. 5** Degree of hypoxia 8% $O_2$,6h -induced apoptotic cell death TUNEL staining in parietal cortex **B** and hippocampus **C** of 7-day old mice upon reoxygenation period of 72 h compared to normoxic controls **D, E** . Quantification of TUNEL+ cells in the parietal cortex **F** upon reoxygenation R of 24 h, 72 h and 7 d N=3 per group compared to controls. *Scale bars* in **B**-**E**: 100 μm; * p<0.05, ** p<0.01

**Fig. 6** Immunohistochemical analysis of Ngb in mouse cerebral cortex at P0 **A** and P7 **C** under normoxic and hypoxic 8% $O_2$, 6h; **B**, **D** conditions. Merged photomicrographs of Ngb detected with Alexa Fluor 488, *green* and NeuN detected with Alexa Fluor 594, *red* are shown. Nuclei are counterstained with DAPI *blue* . An increased number of Ngb+ cells was detectable during acute hypoxia **B** compared to normoxia **A** at P0. In contrast, this hypoxia-induced increase was not found in cortices of 7-day-old mice **D** in comparison to controls **C** .

**Fig. 7** Effects of PHI FG-4497 on Ngb and HIF activation in embryonic mouse neurons E14, DIV6 under normoxic and hypoxic 1% $O_2$ conditions. **A** Up-regulation of Ngb mRNA levels normalized to β-actin in normoxic FG-4497-treated and hypoxic non-treated neurons compared to controls without additive effects in FG-4497-treated hypoxic cells. **B** Western blot analysis and densitometric quantification of HIF-1α protein in neurons exposed to indicated concentrations of FG-4497 under normoxic and hypoxic conditions. 2,2´-dipyridyl was used as a positive control. **C, D** Up-regulation of BNIP3 and ADM mRNA levels in normoxic FG-4497-treated neurons and in non-treated hypoxic neurons compared to controls. **p<0.01; ***p<0.001

**Table 1.** Gene expression of HIF-dependent and HIF-independent factors in developing mouse brain exposed to chronic hypoxia and chronic hypoxia followed by a 24h reoxygenation interval R24h in comparison to controls mean ± SEM .

| **mRNA levels normalized to β-actin** | **normoxia** | **chronic hypoxia** | **normoxia + R24h** | **chronic hypoxia + R24h** |
|---|---|---|---|---|
| | n=5 | n=3 | n=5 | n=3 |
| Ngb | 1.80±0.22 | 2.01±0.05 | 0.40±0.18 | 0.82±0.35 |
| BNIP3 | 1.24±0.07 | 1.40±0.08 | 0.53±0.07* | 1.24±0.19* |
| ADM | 0.73±0.03 | 0.81±0.04 | 0.38±0.08 | 0.57±0.07 |
| EPO | 0.06±0.01 | 0.74±0.09** | 0.01±0.00 | 0.08±0.04** |
| PHD2 | 0.78±0.23 | 1.14±0.05 | 0.16±0.05 | 0.32±0.13 |
| PHD3 | 0.73±0.03 | 0.81±0.04 | 0.43±0.05 | 0.57±0.07 |
| DUSP1 | 1.61±0.32 | 1.46±0.12 | 0.37±0.07 | 0.43±0.25 |
| IER3 | 0.86±0.12 | 1.01±0.11 | 0.65±0.05 | 0.70±0.08 |

* $p < 0.05$; ** $p < 0.01$

**Table 2.** Gene expression of Ngb, BNIP3 and ADM in developing mouse brain at P0 and P7 n=4 upon FG-4497 treatment 30-100 mg/kg; i.p.; 6h in comparison to controls mean ± SEM .

| **mRNA levels normalized to β-actin** | **Ngb** | | **BNIP3** | | **ADM** | |
|---|---|---|---|---|---|---|
| | P0 | P7 | P0 | P7 | P0 | P7 |
| non treated | 0.18±0.07 | 0.41±0.08 | 0.20±0.04 | 0.52±0.17 | 0.06±0.01 | 0.05±0.01** |
| NaCl 0.9 % | 0.37±0.03 | 0.65±0.07 | 0.18±0.03 | 0.58±0.04 | 0.03±0.01** | 0.06±0.01 |
| PHI, 30 mg/kg | 0.30±0.06 | 0.48±0.06 | 0.28±0.06 | 0.62±0.08 | 0.11±0.01** | 0.14±0.03 |
| PHI, 60 mg/kg | 0.28±0.05 | 0.35±0.25 | 0.25±0.01 | 0.77±0.36 | 0.09±0.01 | 0.11±0.02 |
| PHI, 100 mg/kg | 0.42±0.15 | 0.21±0.09 | 0.22±0.03 | 0.53±0.12 | 0.09±0.01 | 0.29±0.04** |

** $p < 0.01$ vs controls

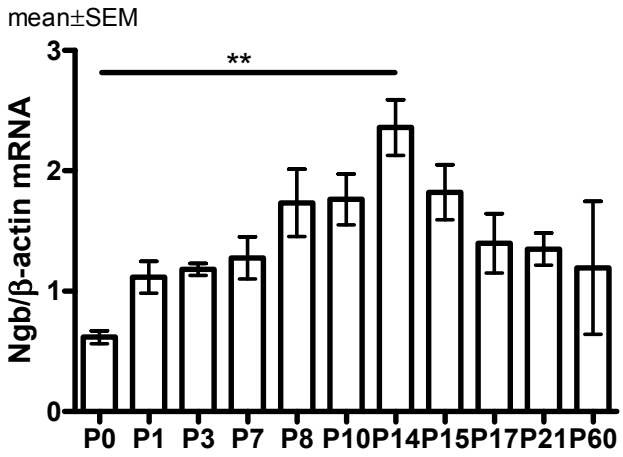

mean±SEM
**
Ngb/β-actin mRNA
3
2
1
0
P0
P1
P3
P7
P8
P10
P14
P15
P17
P21
P60

**Fig. 1**

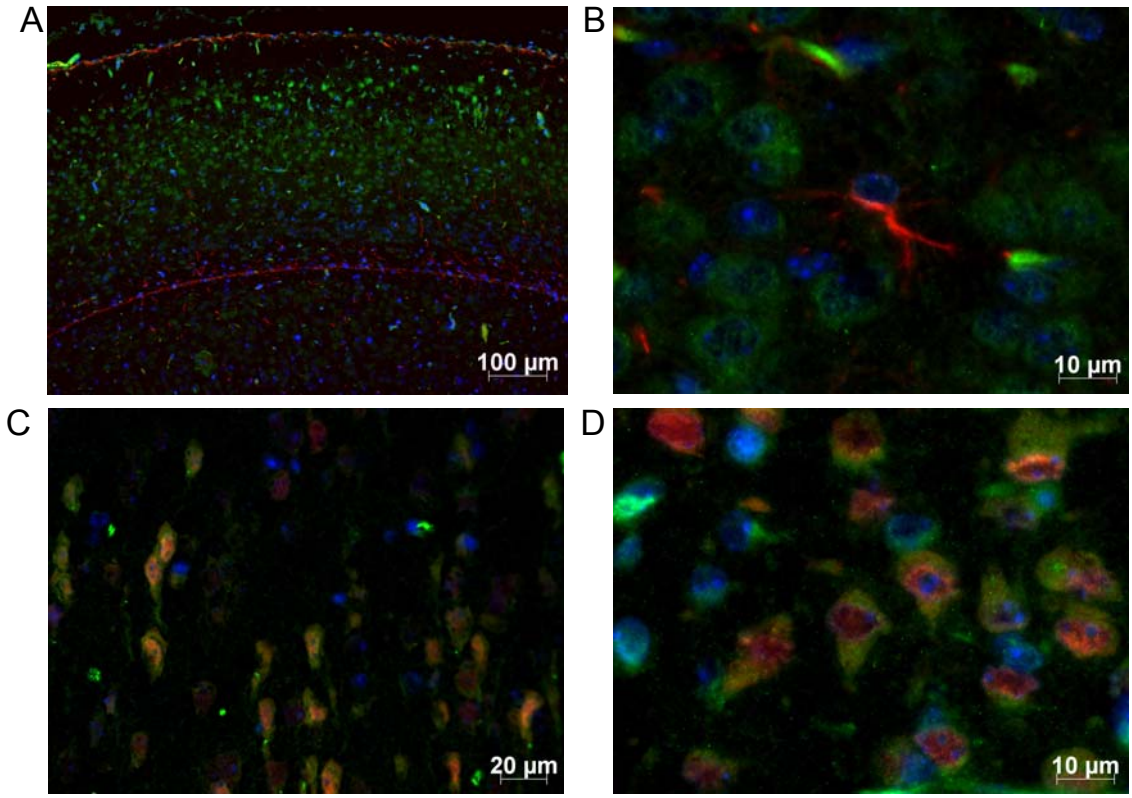
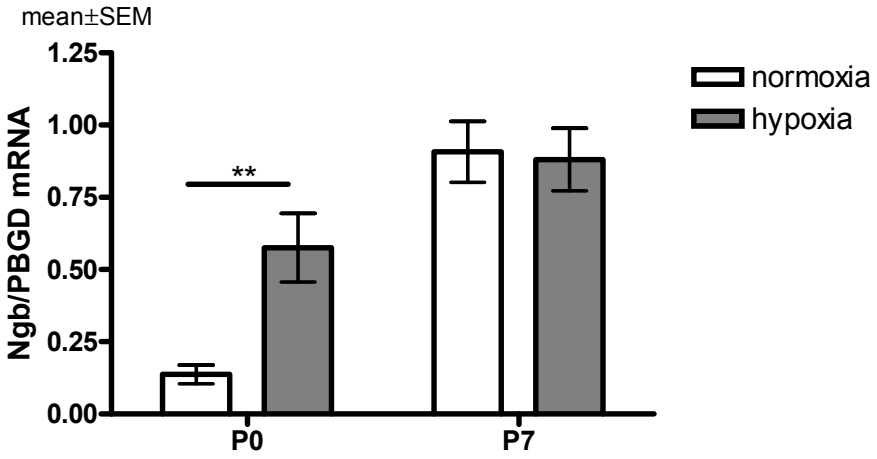



**Fig. 2**

**A**


mean±SEM
1.25
1.00
0.75
0.50
0.25
0.00
Ngb/PBGD mRNA
**
P0
P7
normoxia
hypoxia

**B**

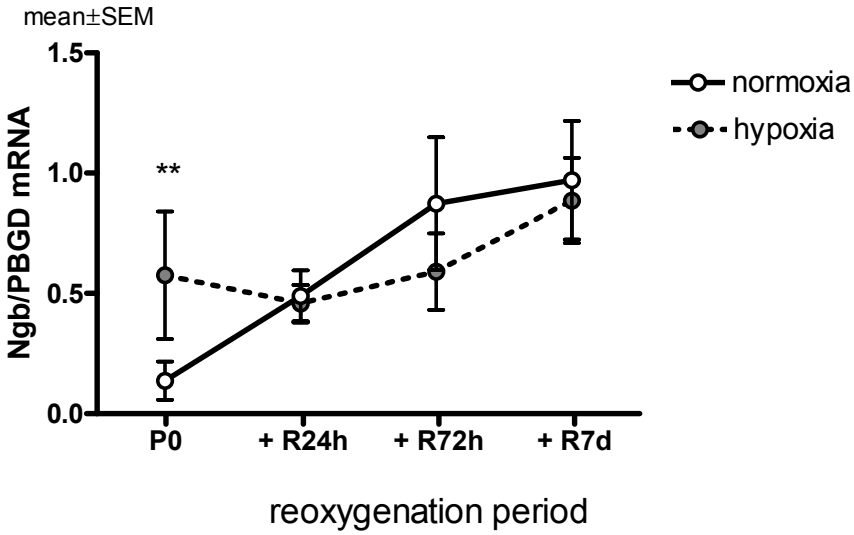

mean±SEM
1.5
1.0
0.5
0.0
Ngb/PBGD mRNA
**
P0
+ R24h
+ R72h
+ R7d
reoxygenation period
normoxia
hypoxia

**Fig. 3**

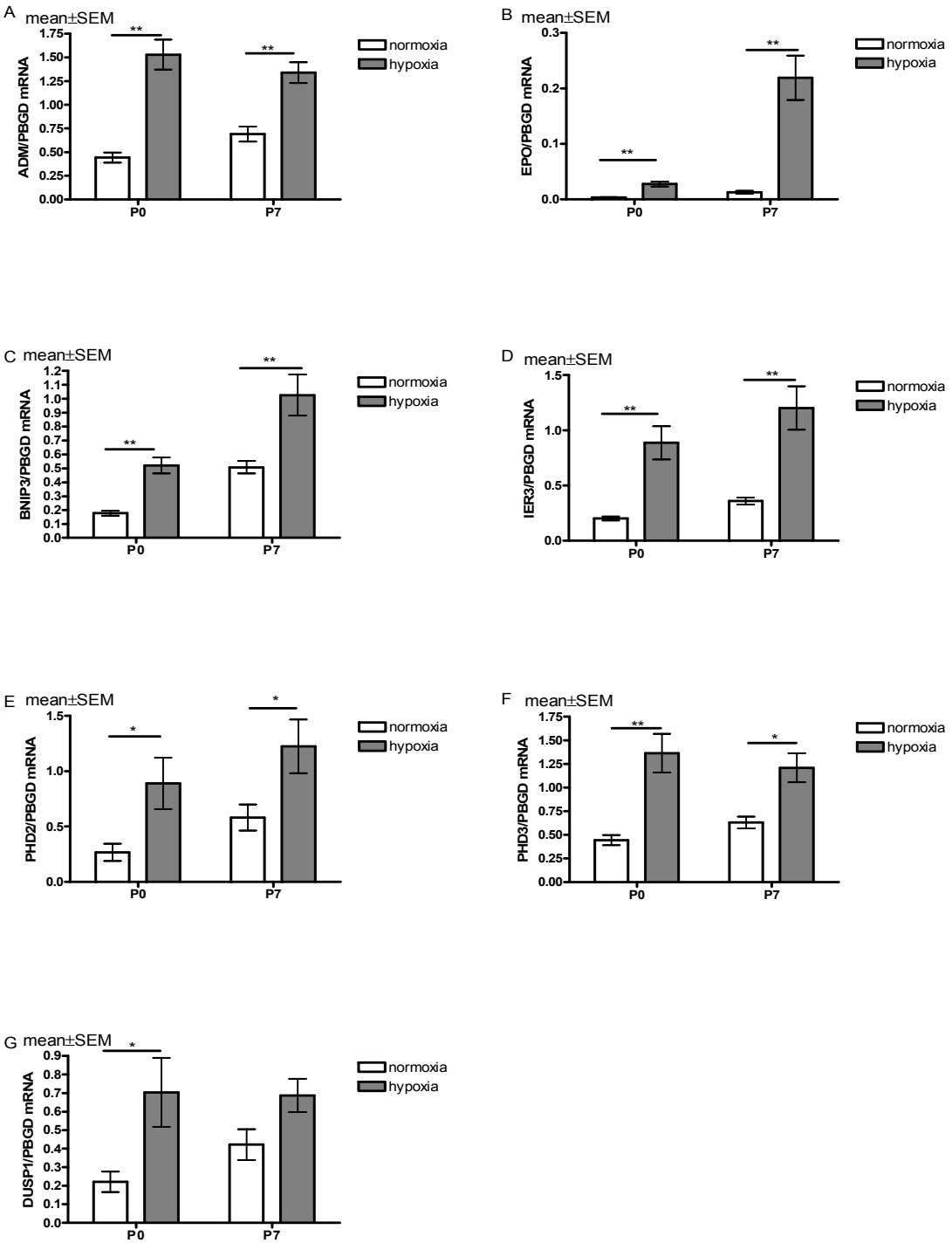
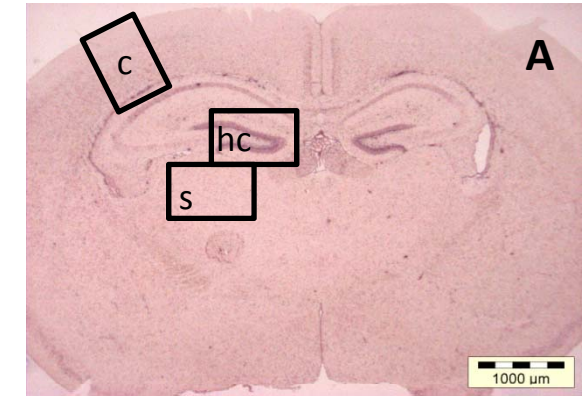
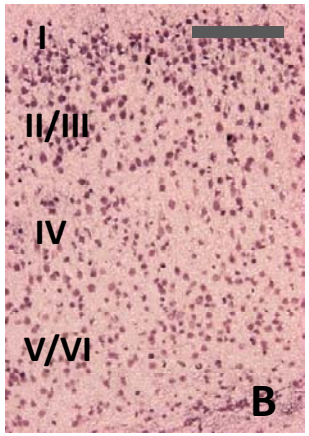
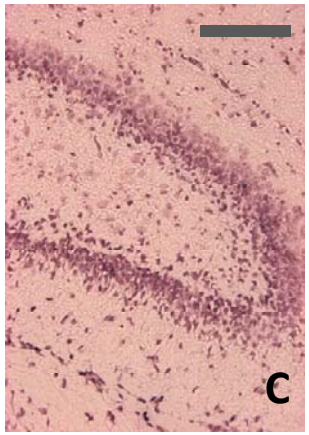
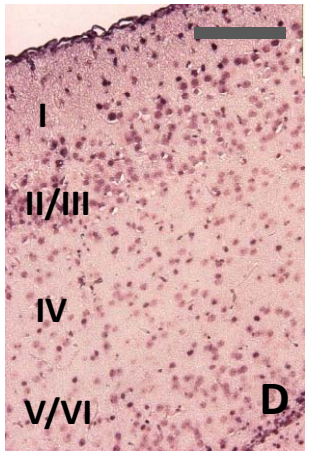

A mean±SEM
ADM/PBGD mRNA
normoxia
hypoxia
P0
P7
B mean±SEM
EPO/PBGD mRNA
C mean±SEM
BNIP3/PBGD mRNA
D mean±SEM
IER3/PBGD mRNA
E mean±SEM
PHD2/PBGD mRNA
F mean±SEM
PHD3/PBGD mRNA
G mean±SEM
DUSP1/PBGD mRNA

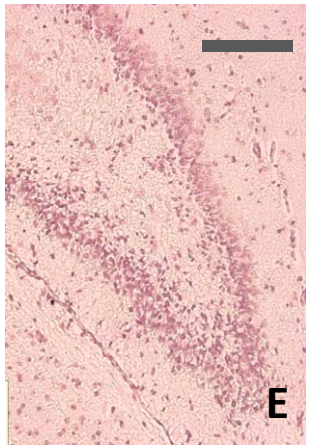
**Fig. 4**



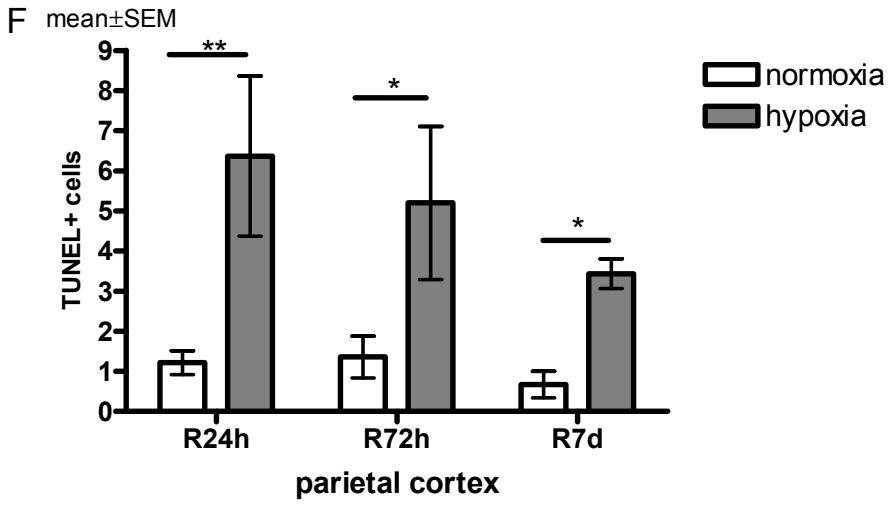




Cortex (c) Hippocampus (hc)


F mean±SEM
TUNEL+ cells
0
1
2
3
4
5
6
7
8
9
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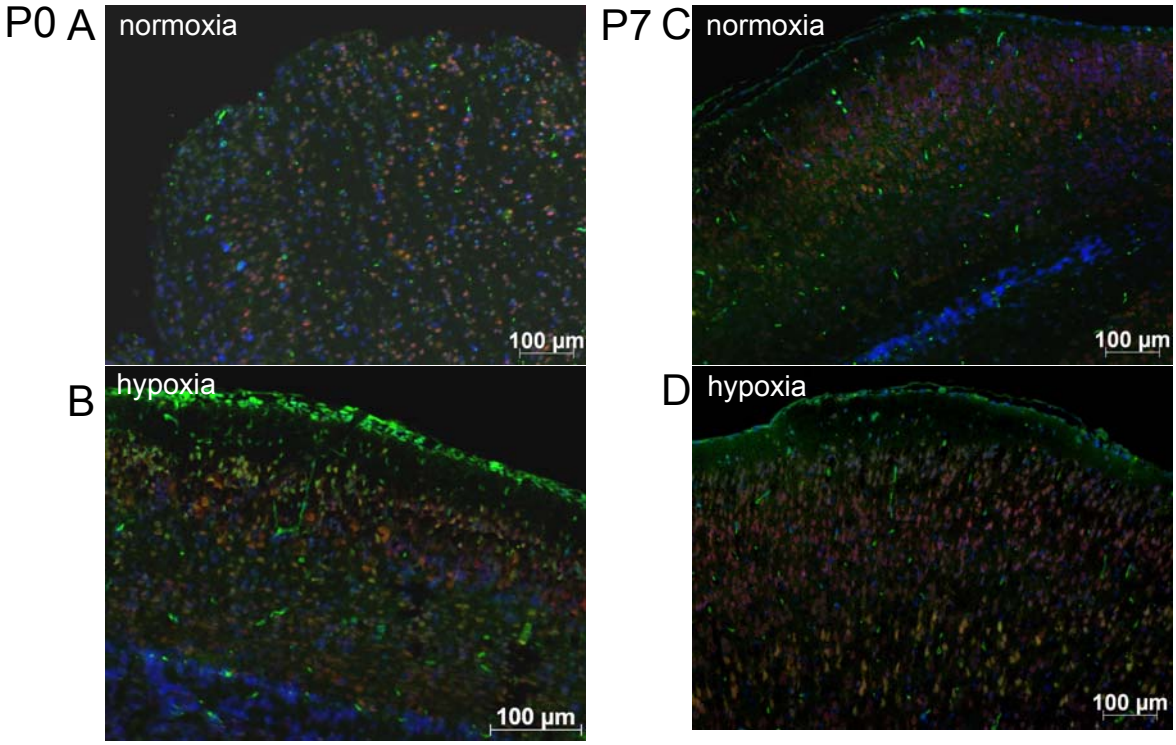
*
*
R24h
R72h
R7d
parietal cortex
normoxia
hypoxia

***Fig. 5***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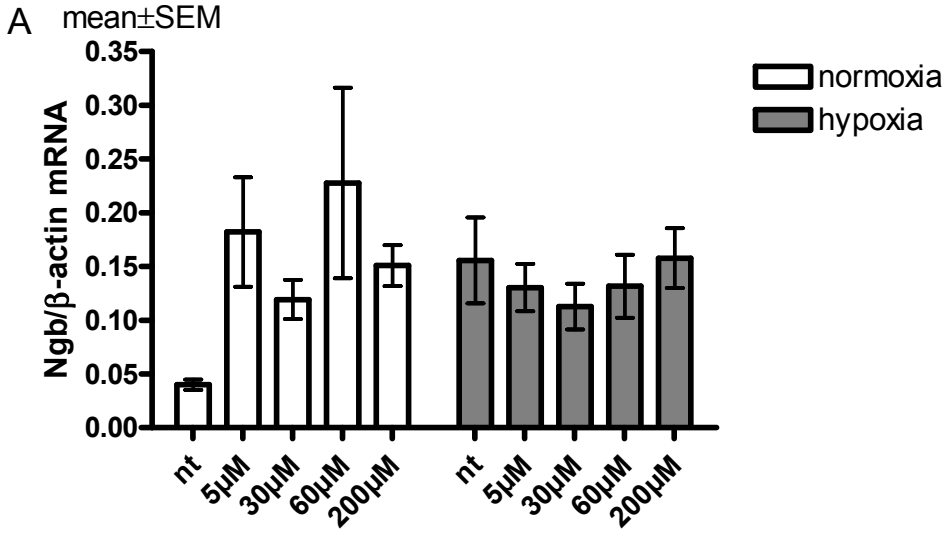
normoxia
100 µm
B
hypoxia
100 µm
P7 C
normoxia
100 µm
D
hypoxia
100 µm


A mean±SEM
Ngb/β-actin mRNA
0.35
0.30
0.25
0.20
0.15
0.10
0.05
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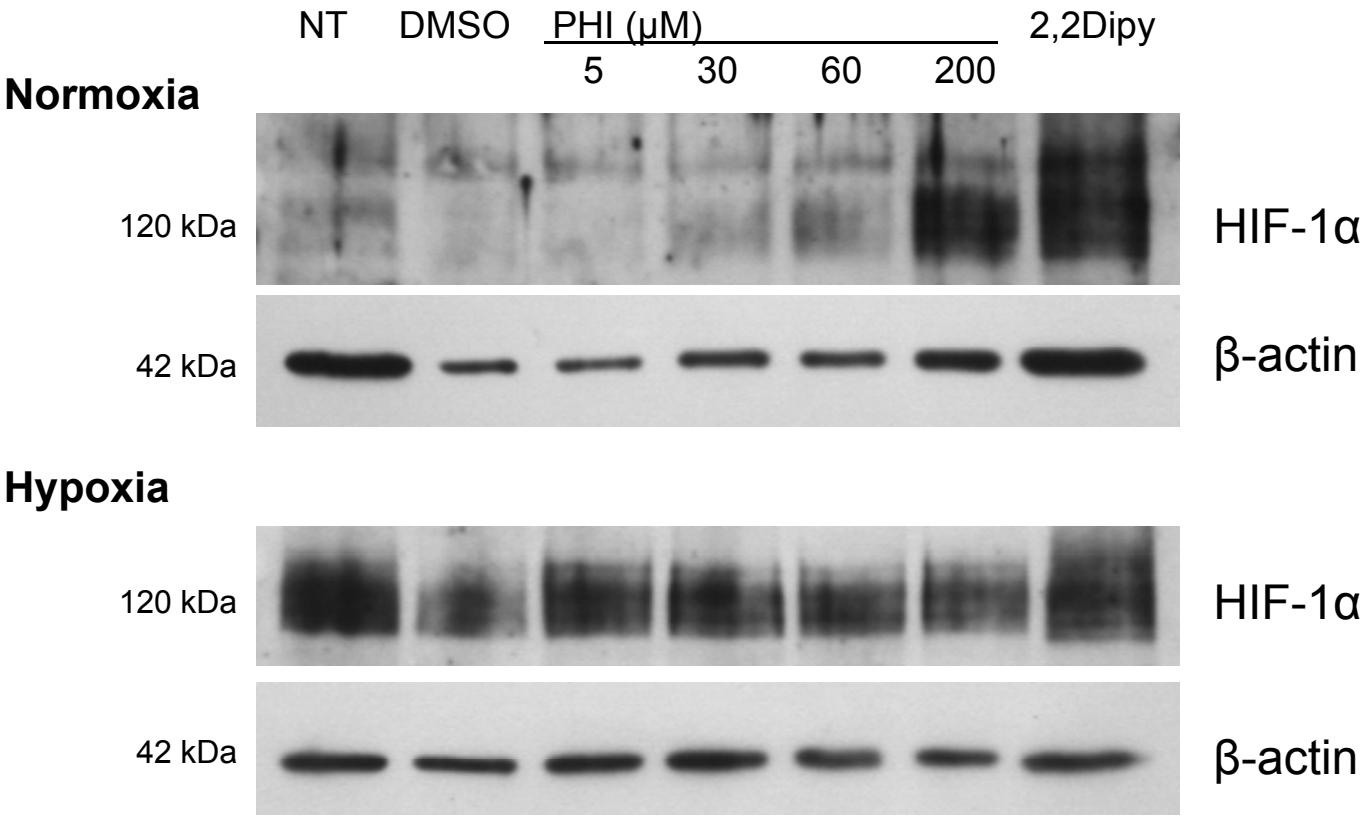
nt
5μM
30μM
60μM
200μM
nt
5μM
30μM
60μM
200μM
normoxia
hypoxia

***Fig. 7A***


NT
DMSO
PHI (μM)
5
30
60
200
2,2Dipy
Normoxia
120 kDa
HIF-1α
42 kDa
β-actin
Hypoxia
120 kDa
HIF-1α
42 kDa
β-actin

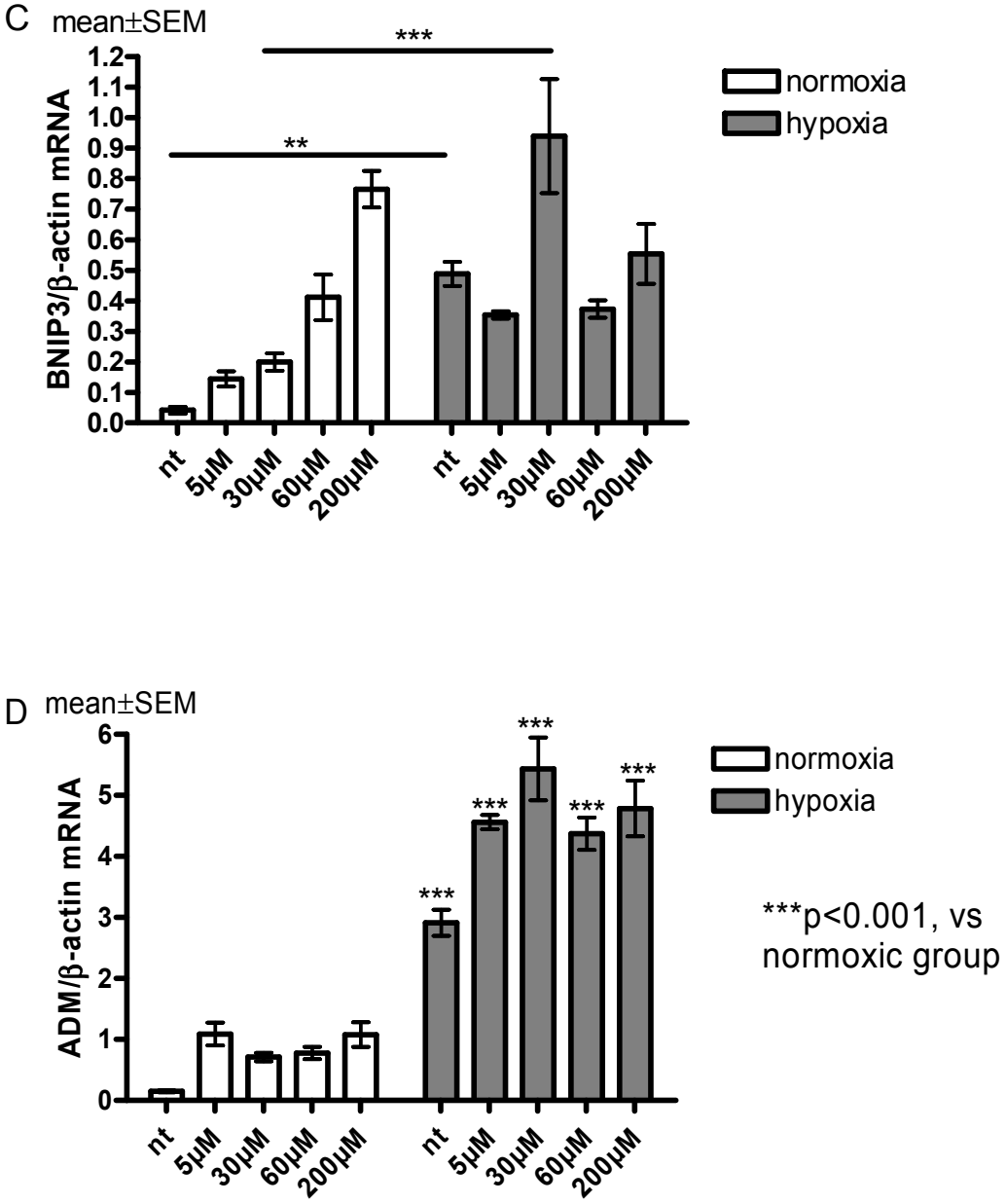

C mean±SEM
BNIP3/β-actin mRNA
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
**
***
normoxia
hypoxia
nt
5μM
30μM
60μM
200μM
nt
5μM
30μM
60μM
200μM
D mean±SEM
ADM/β-actin mRNA
6
5
4
3
2
1
0
***
***
***
***
***
normoxia
hypoxia
***p<0.001, vs
normoxic group
nt
5μM
30μM
60μM
200μM
nt
5μM
30μM
60μM
200μM

***Fig. 7 C-D***